#4

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

John Victor LAMONT et al. Group Art Unit: 1641

Application No.: 10/046,728

Filed: January 17, 2002 Docket No.: 111723

For: IMAGING METHOD

CLAIM FOR PRIORITY

Director of the U.S. Patent and Trademark Office
Washington, D.C. 20231

Sir:

The benefit of the filing date of the following prior foreign application filed in the following foreign country is hereby requested for the above-identified patent application and the priority provided in 35 U.S.C. §119 is hereby claimed:

Great Britain Patent Application No. 0102357.1 filed January 30, 2001.

In support of this claim, a certified copy of said original foreign application:

__X__ is filed herewith.

_____ was filed on _____ in Parent Application No. _____ filed _____.

_____ will be filed at a later date.

It is requested that the file of this application be marked to indicate that the requirements of 35 U.S.C. §119 have been fulfilled and that the Patent and Trademark Office kindly acknowledge receipt of this document.

Respectfully submitted,

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Date: April 2, 2002

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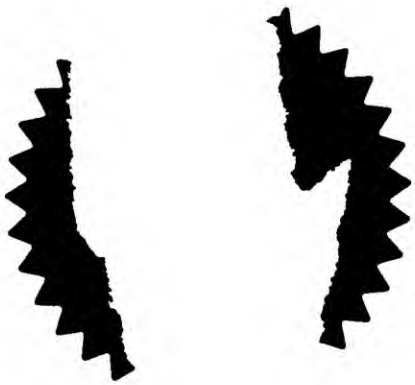

Signed

Dated 26 February 2002

Patents Form 1/77

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1/77

Request for grant of a patent

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The Patent Office

Cardiff Road
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NP9 1RH

1. Your reference — REP06450GB — 31JAN01 E601957-1 D02890 P01/7700 0.00-0102357.1

2. Patent application number *(The Patent Office will fill in this part)* — 30 JAN 2001 — 0102357.1

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Randox Laboratories Ltd.
Ardmore
Diamond Road
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BT29 4QY

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

GB0026800.1

4. Title of the invention — Imaging Method

5. Name of your agent *(if you have one)* — Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* — 745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
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Claim(s)	2
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	NO

11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date
30 January 2001

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward
020 7377 1377

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IMAGING METHOD

Field of the Invention

This invention relates to a device and apparatus for performing multi-analyte assays.

Background of the Invention

There is now widespread interest in the use of fabricated arrays of molecules in the detection and characterisation of analytes. For example, fabricated arrays of polynucleotides are now used widely in DNA sequencing procedures and in hybridisation studies for the detection of genetic variations in a patient.

The fabricated arrays can be designed to include high densities of the same or different molecules immobilised on a solid support surface. This allows the user to generate many results in one experimental procedure. The arrays also have the advantage in that the analytical methods can be automated, thereby allowing a high throughput of samples to be achieved.

The arrays are usually designed with a plurality of individual reactions sites located in spatially-distinct areas on a solid support. In order to produce the arrays in spatially-distinct areas, the most common approach has been through photolithographic techniques. The solid support is coated with a photolabile linker, which only becomes reactive towards a binding ligand following irradiation with light of a suitable wavelength. Spatial resolution is achieved by placing a physical mask on the solid support surface. The pattern of holes in the mask determines the pattern of binding regions on the solid support.

WO-A-95/16204 describes a photolithographic approach using avidin and the photolabile molecule photobiotin. Spatial resolution has also been achieved by passive adsorption. For example, US-A-5432099 discloses binding of the molecules to the solid support surface through a combination of ionic interactions, hydrophobic interactions and Van Der Waals forces.

One particular example of fabricated arrays concerns solid support materials immobilised with nucleic acids. These arrays consist typically of a high-density matrix of polynucleotides immobilised in spatially-distinct regions. Fodor *et al* Trends in Biotechnology (1994) 12:19-26, describes ways of assembling the nucleic acids using a chemically sensitised glass surface protected by a mask, but exposed at defined areas to allow attachment of suitably modified nucleotide phosphoramidites. Stimpson *et al*, PNAS (1995) 92:6379-6383, describes the manufacture of fabricated arrays by

the technique of "spotting" known polynucleotides onto a solid support at predetermined positions.

In order to maximise the potential and the sample throughput using the array technology, it is essential to fully automate the signal processing from the reaction sites of the arrays. Therefore, it is necessary to perform the imaging of the array, and further mathematical processing of that image, without manual intervention. A difficulty that is experienced with fully automated systems is that the location of each reaction site is sometimes difficult to locate accurately. This may be due to the nature of the array manufacturing process, where it is not possible for the array to be in exactly the same position on each device. The problems may also be due to slight movement of the devices in the apparatus, which may be caused during the washing steps necessary in the assay procedure. As each distinct reaction site may be separated by a distance of only 10-50 μm, it is difficult to ensure that the automated system has accurately positioned each reaction site. For example, movement of the array can be of the same order of magnitude as the distance between each reaction site. This means that predefined windows in the image, for each reaction site to be analysed, cannot be easily used, as the movements may cause the wrong reaction site to fall inside an inspection window which defines the boundary of the reaction site. Alternatively, a reaction site may be missed by the windows, or possibly two sites may fall within the same window. To make processing easier, it is necessary to ensure that only one reaction site falls wholly within each analyte inspection window.

There is therefore a need for improved method for devices which allow accurate positioning of each discrete reaction site on an array.

Summary of the Invention

The present invention is based on the realisation that accurate positioning of individual reaction sites on an array can be carried out by incorporating a reference molecule on each array.

According to a first aspect of the invention, a method for imaging molecules contained in an array of discrete reaction sites on the surface of a solid support, comprises,

(i) imaging the array and detecting a first molecule located on the solid support at a known position with respect to the array;

(ii) by reference to the first molecule, aligning inspection windows in registration with the discrete reaction sites; and

(iii) determining the amount of detectable signal in each window.

The method allows accurate positioning of the inspection windows, thereby providing improvements to conventional automated systems.

As the first molecule is in a known position on the solid support with respect to the array, it is possible to align the inspection windows to be in registration with the arrayed reaction sites.

Description of the Invention

The present invention makes use of conventional apparatus to accurately image arrayed molecules. Using a conventional imaging apparatus, the solid support or "biochip" is imaged using, for example, an optical microscope or a charge-coupled device. The image is digitised by camera electronics into (for example) 512 x 512 pixels, of 16 bits per pixel, and transferred to a computer with an analyser, for processing. In order to accurately position each reaction site on the biochip, the reference molecule (or first molecule) is first located and an array of analyte inspection windows established with respect to the reference molecule (figure 1). The positioned inspection windows are then used to find the reaction sites or "analyte spots" on the biochip.

The reference molecule may also be used as an on-chip quality control check, so that, if the intensity of the detectable signal is outside of a pre-defined range, e.g. because of biochip preparation, the biochip results may be rejected.

The reference molecule should be located at pre-defined positions on each biochip. For example, reference molecules may be located in one or more corners of the biochip. Therefore, one or more inspection windows may be defined in fixed locations, corresponding to the expected reference molecule locations. The manufacturing tolerances are such that the reference molecules can fall within somewhat larger search inspection windows than the reaction site or analyte inspection windows, even though one or more reaction sites from the array may also fall inside the reference molecule inspection window. The search strategy, used to find only each reference molecule in the inspection window, and ignoring other reaction sites must be chosen to ensure that the reference is found first. For example, if a reference molecule is located in the top left corner of each biochip, then a diagonal search, starting in the top left corner of the inspection window, will encounter the reference molecule first, and so allow it to be correctly identified (figure 2). For other reference locations, the search strategy can be adjusted accordingly. Searching may

be carried out using conventional software to move the search inspection window across the solid support to locate and be in registration with the reference molecule.

Once the reference molecule position is identified, the analyte inspection windows may be positioned with respect to it (figure 3). This is aided either by the use of a second reference molecule located at a known position or by the knowledge that the first reference molecule is positioned in a fixed position with respect to the array of reaction sites. Locating the reference molecule allows the position of the array to be determined.

The coordinates of the reference molecule are used to position the inspection windows for the discrete reaction sites containing the molecules to be analysed. Processing of the signal determines the amount of detectable signal, and thereby the level of reaction at that site. Processing may be carried out using conventional techniques, including confocal microscopy or charge-coupled devices (CCD). In a preferred embodiment, the biochip device is used in a system having a charge-coupled device to visualise the arrays.

The image may be processed as follows. The imaging device is used to generate an image which is copied to a processor. Several conventional morphological smoothing operations may then be performed on it, to generate a smoothly varying background image. This is then subtracted from the original image to give the background-corrected image. A conventional thresholding operation may be performed on the image data, to create a binary image, by segmenting the image into white, for those areas above the threshold, and black for those below the threshold, where the threshold is chosen at an appropriate level to finding the reference molecules.

The segmented reference molecules should form discrete contiguous 'blobs'. 'Blob' analysis, a term used conventionally to describe the processing of such discrete segmented regions, is used to find those pixels which form part of the reference molecules, within a pre-defined inspection window. 'Blob' analysis, typically, uses a contour-following operator to follow the outline of a segmented region, so that a closed boundary is formed. Common in digital image processing, or contour following, is the chain code or Freeman code, which dates from 1961. This code, and variants thereof, may be used to calculate the perimeter and size of a segmented object, and such parameters may be used to verify that the size and shape of the segmented blobs fall within the expected limits for the reference molecules. If the 'blob' is too small, too

large, or the wrong shape (e.g. ratio of length to height, or circularity), it may be rejected - this allows for cosmic ray artefacts, stray reflections from the biochip walls, sections of other analyte spots intruding into the window, etc., to be ignored.

Commercial programs using 'blob' analysis include IMAQ Vision Software from National Instruments, and CVC from Stemmer Imaging - other programs, such as Neurocheck4.2, from Data Translation Ltd., use a different terminology (referring to regions of interest rather than blobs), but perform essentially the same operations on the pixel data for measurements of areas found by various search strategies.

For a reference molecule located in or near the corner of a biochip analyte array, a diagonal search of the binarised reference inspection window (in a direction appropriate to ensuring that the reference molecule is found first), would be used to locate the first pixel that falls above the threshold, along the diagonal line, and the connectedness to other above-threshold pixels may be determined in the vicinity of this pixel. If it is connected directly to a sufficient number of other above-threshold pixels, it may be considered to form part of the boundary of the reference molecule. The search continues to locate all above-threshold pixels forming part of the boundary, and the chain code is used to close the boundary, starting and finishing at the first boundary pixel, and, thus, identifying only the pixels that form the reference molecule.

The pixels that are found to fall on or within the closed boundary, can then have their central positions calculated for each reference window, and these central positions can then be used to define the locations of the analyte inspection windows. When the analyte inspection window positions are determined, the image data within these may be analysed similarly to determine those pixels that form each of the discrete test reaction sites (DTRs), which determine the discrete regions of arrayed molecules (reaction sites). The background-corrected image intensities for each of these DTR segmented 'blobs' may then be processed further to calculate the DTR signal at each of these locations and, therefore, the degree of biochemical reaction that has occurred at each DTR site.

Typically, to calculate the DTR signal for those pixels identified for each DTR site, the maximum intensity for each site is found, and then all pixels having an intensity within an empirically determined range, e.g. 20% below each maximum, will be used to measure the DTR signals. Such a DTR signal measurement may be a simple summation of all pixel intensities, within the previous limits, or, possibly, an

average thereof, for each DTR site. An example of a preferred imaging process is disclosed in EP-A-0902394.

The array of discrete reaction sites may not always be aligned in a regular pattern. To correct for any misalignments, it may be preferable, once the reference molecule has been located, to align an analyte inspection window so that all the possible reaction sites are located within the one window. Imaging using blob analysis will reveal the closed contours of the reaction sites and will permit an individual inspection window to be aligned for each reaction site, so that the central positions can then be calculated.

The solid support material which is used in a device of this invention may be, for example, silicon, plastic, membrane forming materials, quartz, glass or ceramic materials (aluminium oxide). Ceramic materials provide an excellent alternative to silicon, since both fluorescent and chemiluminescent detection techniques can be employed successfully.

The solid support material used in the invention may be less than 1 cm^2. The discrete regions of immobilised molecules may be separated by less than 200 μm, preferably less than 100 μm, and most preferably 10-15 μm.

Preferred devices which may be used in the invention may be found in GB-A-2324866.

The molecules used in the invention, may be immobilised on the surface of the material using conventional means. Covalent immobilisation is preferred. Passive adsorption may also be used, but this form of immobilisation is susceptible to changes in pH, temperature and ionic strength, and may in some instances result in release of weakly-bound molecules during incubation and washing steps, thus contributing to poor reproducibility. It is of course desirable that the molecules retain maximum activity, after the immobilisation procedure.

Covalent immobilisation of the molecules may be carried out using conventional techniques, typically using a chemically-reactive linker molecule, which can be activated under defined conditions. Examples of suitable linker molecules are described in GB-A-2324866.

The molecules immobilised to the solid support material may be any suitable for use in an analyte assay. For example, the arrayed molecules may be polynucleotides, e.g. DNA, RNA, or functional analogues thereof. Alternatively,

proteins and peptides may be used, e.g. enzymes, antibodies, receptors or hormones. The molecules may also be viruses or an organic compound.

The reference molecule may be visualised by various techniques, including colorimetric, chemiluminescent, fluorescent or bioluminescent means. The molecule may therefore be any entity which is capable of generating or facilitating the generation of a detectable signal. In one embodiment, the molecule is a fluorescent label which is bound to the device.

Suitable fluorescent labels will be known to the skilled person. Examples include: rhodamine, CY-5, fluorescein, fluorescein isothiocyanate and oregon green.

The fluorescent label may be attached directly to the solid support using chemical means. Alternatively, the fluorescent label may be attached indirectly via an immobilised linker molecule, e.g. a protein, or antibody molecule, or through hybridisation with complementary polynucleotides. Alternatively, a labelled polynucleotide may be used without the requirement for hybridisation.

Alternatively, the molecule may be a biological molecule capable of interacting with a ligand to generate a detectable signal. An example of a suitable biological molecule is an enzyme, e.g. horseradish peroxidase, luciferase or β-galactosidase. Each of these enzymes is capable of participating in a biological reaction which generates a detectable signal. In an alternative embodiment, an antibody is used as the reference molecule and in use, the antibody binds to a ligand which is itself detectable. For example, the antibody may have affinity for the enzyme horseradish peroxidase, which is able to undergo a chemiluminescent process to generate the detectable signal. Preferably, the reference molecule does not react with the analytes which are brought into contact with the arrayed molecules on the solid support. Furthermore, the reference molecule should always be detectable, irrespective of the other analyte reactions that are carried out on the solid support. It is preferable therefore that the reference molecule is chosen to be independent from those molecules undergoing reaction in the analyte assay.

CLAIMS

1. A method for imaging molecules contained in an array of discrete reaction sites on the surface of a solid support, comprising:

(i) imaging the array and detecting a first molecule located on the solid support at a known position with respect to the array;

(ii) by reference to the first molecule, aligning inspection windows in registration with the discrete reaction sites; and

(iii) determining the amount of detectable signal in each window.

2. A method according to claim 1, wherein detection of the first molecule is carried out by aligning a first inspection window within a region of the support that includes the first molecule and searching within the window for an image of the first molecule.

3. A method according to claim 2, wherein the first inspection window defines a two-dimensional array of pixels and searching is carried out by scanning diagonally the array of pixels and determining values for the pixels.

4. A method according to claim 2 or claim 3, wherein, after detecting the first molecule, the first inspection window is repositioned or enlarged so that one or more of the discrete reaction sites is also located within the window, detecting the one or more sites and, by reference to the first molecule and the one or more sites, aligning a further inspection window in registration with each reaction site of the array.

5. A method according to any preceding claim, wherein the array of reaction sites defines a corner within which the first molecule is located.

6. A method according to any preceding claim, wherein step (i) further comprises detecting a second molecule located on the solid support at a known position with respect to the array, and aligning the inspection windows by reference to both first and second molecules.

7. A method according to any preceding claim, wherein imaging is carried out by detecting emitted radiation.

8. A method according to claim 7, wherein the radiation is chemiluminescent, bioluminescent or fluorescent.

9. A method according to any preceding claim, wherein the molecules of the array are capable of reacting with an analyte.

10. A method according to any preceding claim, wherein the molecules of the array are polynucleotides, antibodies, proteins or organic compounds.

11. A method according to any preceding claim, wherein the solid support is less than 1 cm^2.

12. A method according to any preceding claim, wherein the solid support is a ceramic, silicon or glass material.

13. A method according to any preceding claim, wherein the molecules of the array are covalently attached to the surface of the solid support.

14. A method according to any preceding claim, wherein the image generated in step (i) must be above a pre-defined value in order to proceed with steps (ii) and (iii).

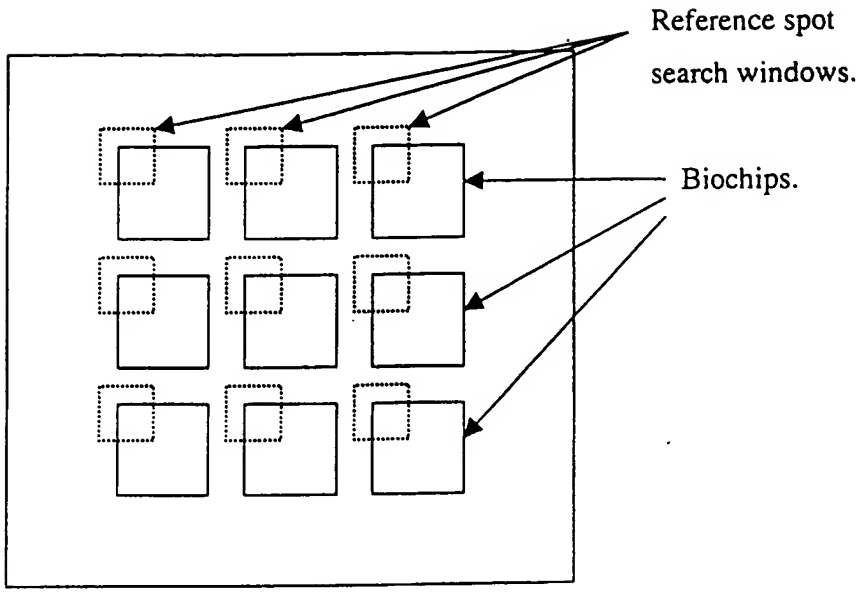

Fig.1 Image representation of biochip array, with reference spot search windows per biochip.

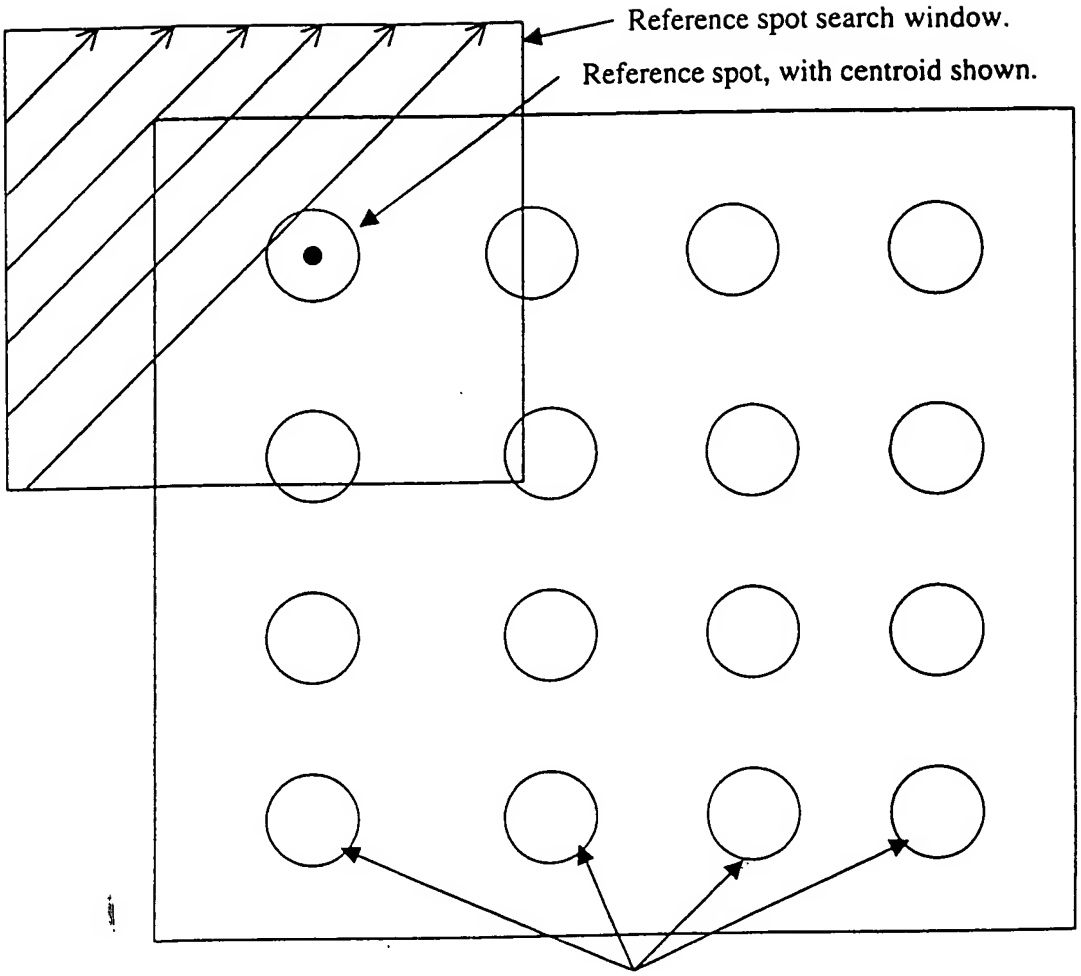

Fig 2. Diagonal search of reference spot window correctly locates reference spot first.

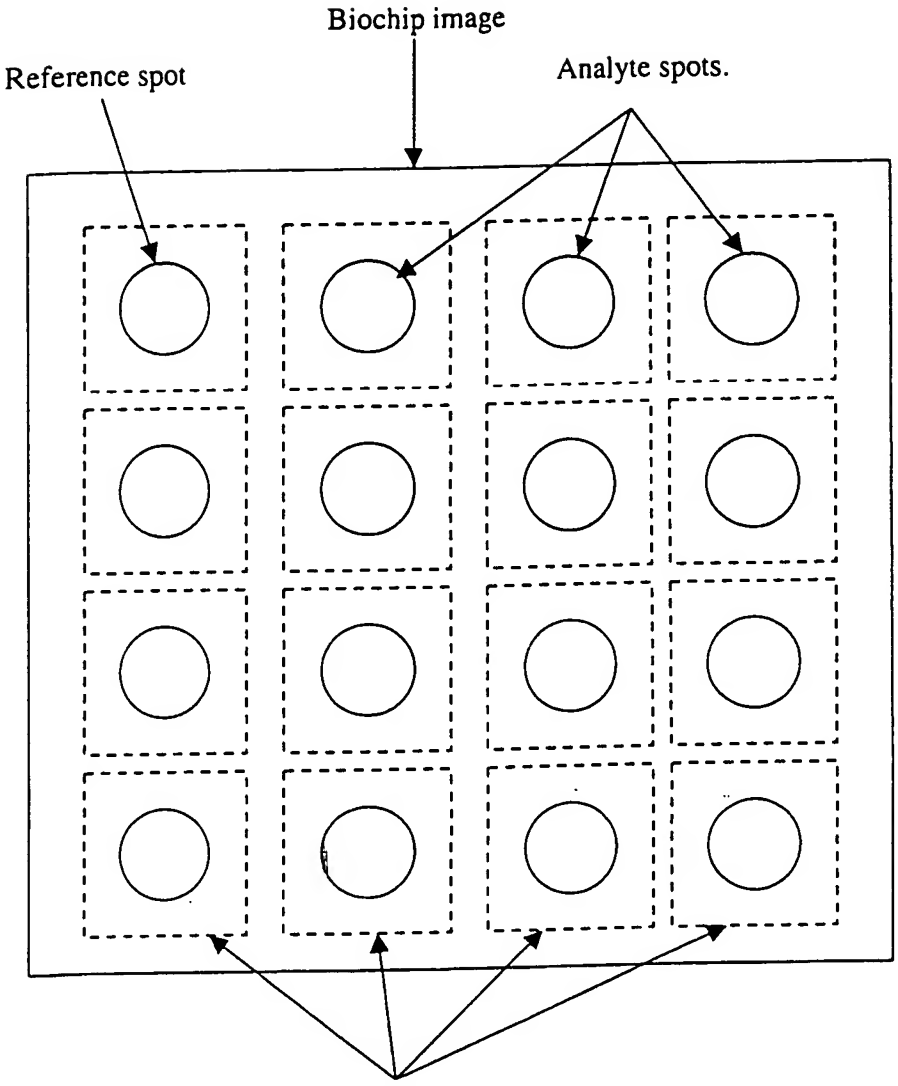

Fig. 3. Analyte windows, for finding analyte spots are located with respect to reference spot centroid.

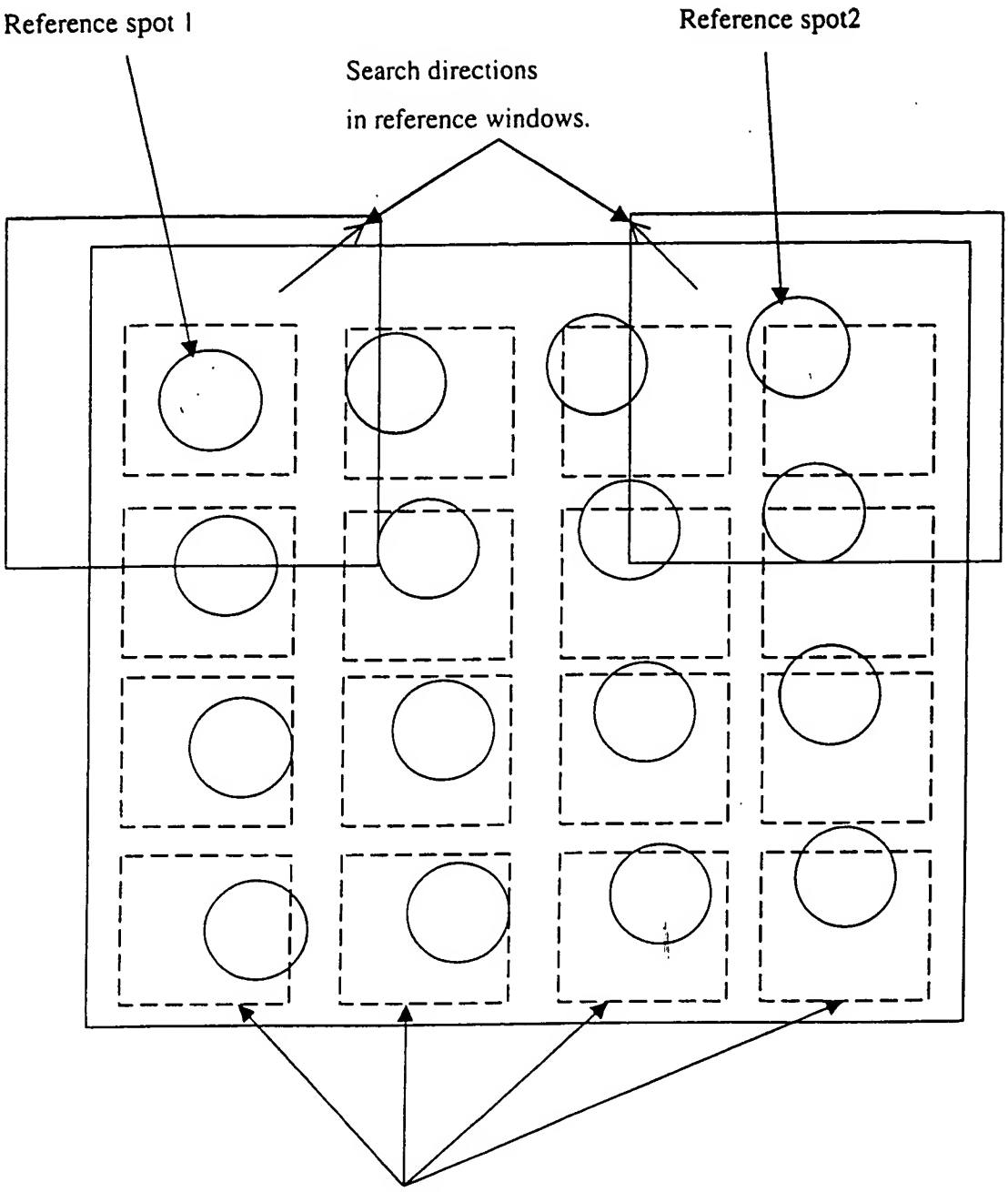

Fig. 4. Biochip rotated in well, showing that, without rotational correction of the analyte inspection windows, as can be calculated from the 2 reference spots, the spots may fall outside the inspection windows. (The window adjustments required are easily calculated, from the angle between the 2 reference spots, see Fig.5.).

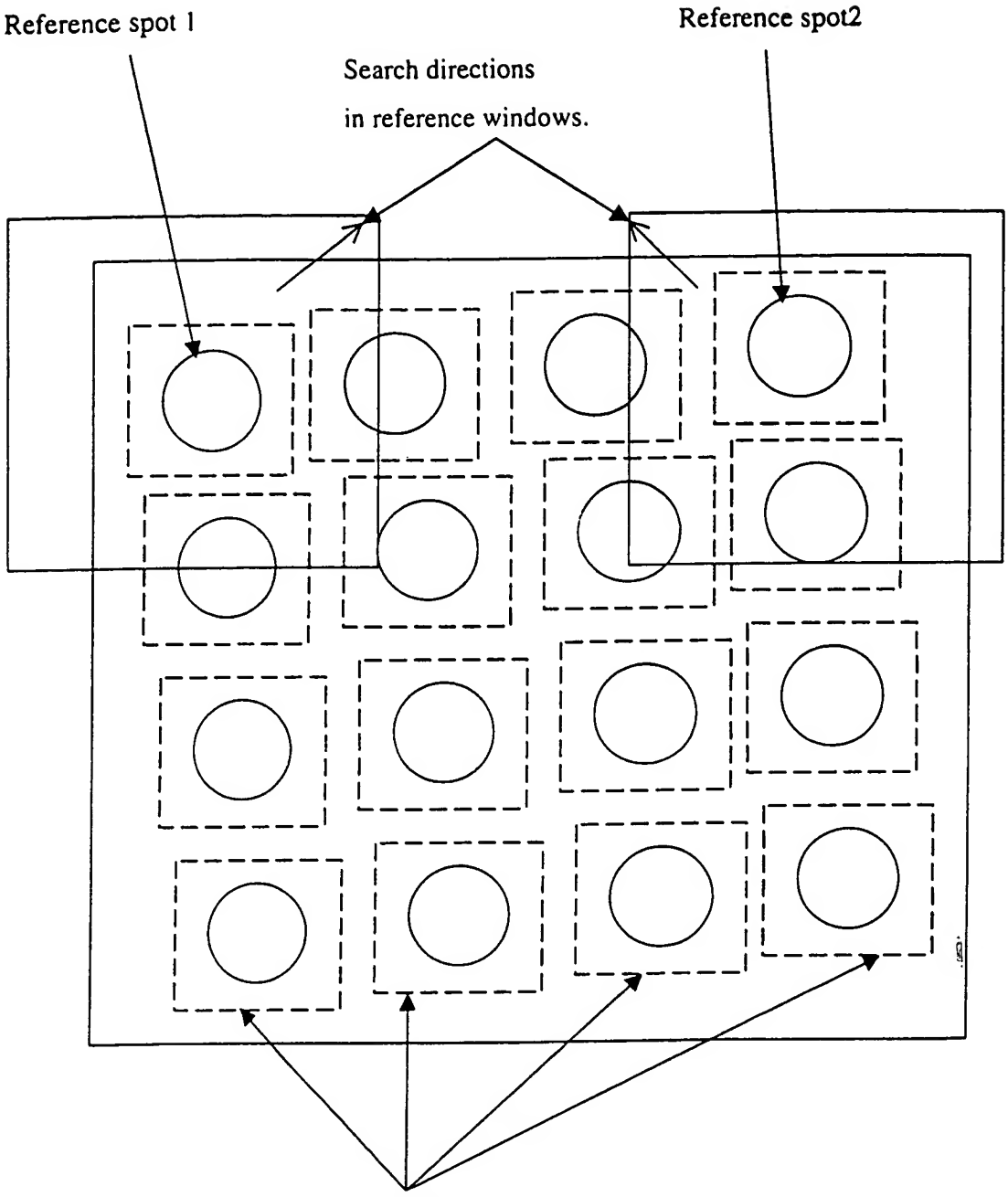

Fig. 5. Biochip rotated in well, with analyte windows re-positioned, by rotational correction from reference spots 1 and2.

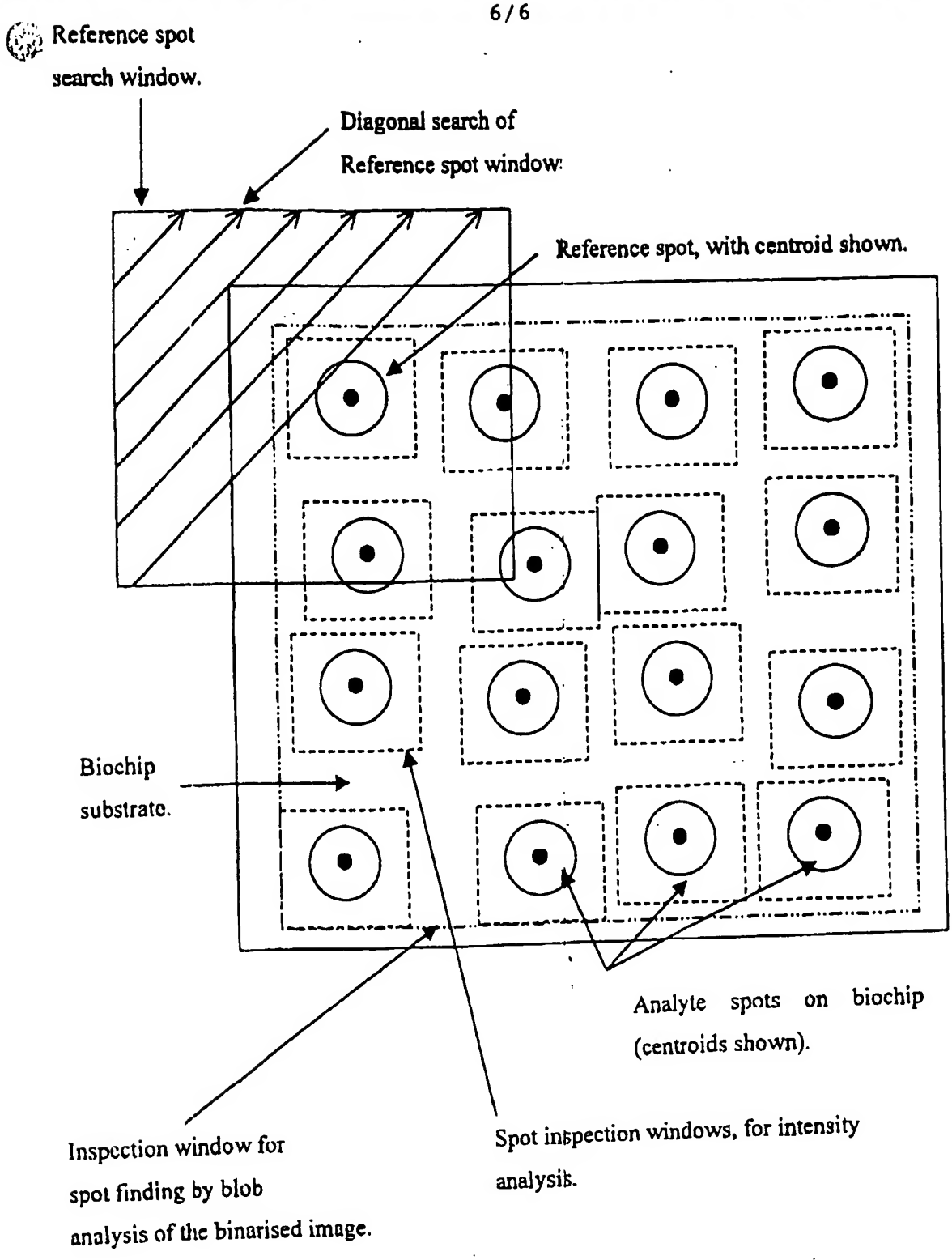

Fig. 3. Further inspection windows, for intensity analysis, on the background-subtracted image. The windows are positioned with respect to the centroids of each spot, as found by blob analysis of the binarised image, in the large inspection window, keyed in position from the centroid of the reference spot, as found first, in the reference spot window.